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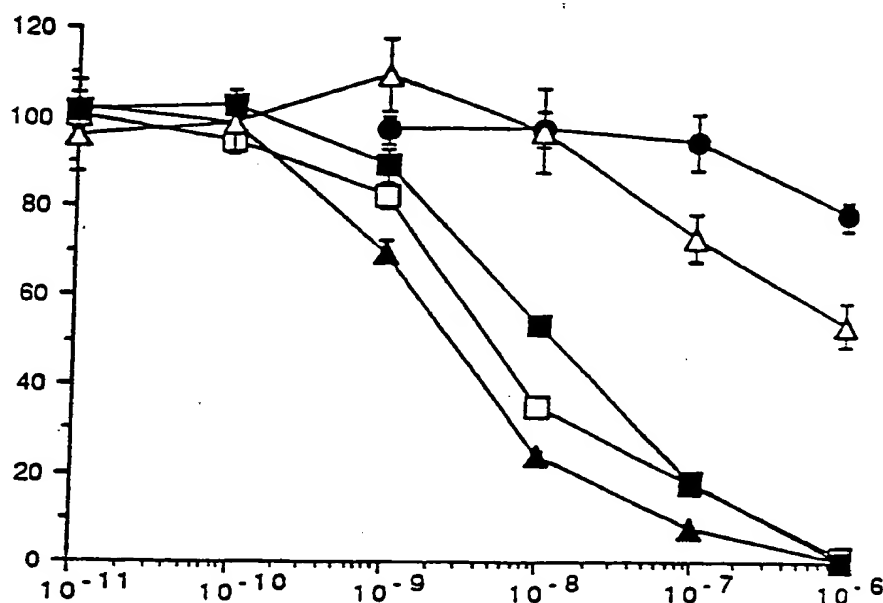
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(54) Title: HUMAN NEUROPEPTIDE Y-Y1 RECEPTOR



(57) Abstract

The invention provides a cDNA sequence and a genomic DNA sequence which encode the human neuropeptide Y-Y1 receptor. These DNA sequences can be used to express the NPY-Y1 receptor in cells to study its agonist and antagonist activity.

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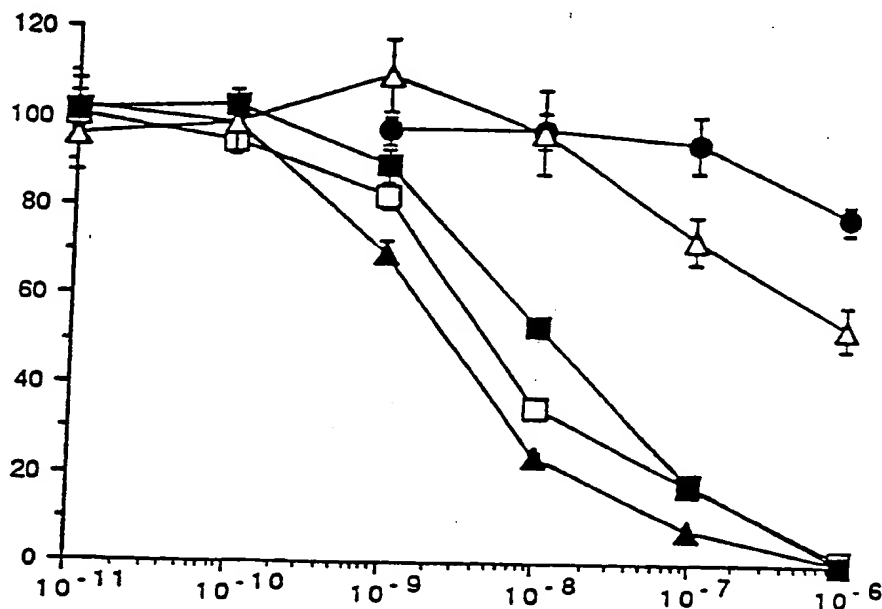
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The invention provides a cDNA sequence and a genomic DNA sequence which encodes the human neuropeptide Y-Y1 receptor. These DNA sequences can be used to express the NPY-Y1 receptor in cells and can be used to screen compounds for neuropeptide Y agonist and antagonist activity.

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ES	Spain	MN	Mongolia	VN	Viet Nam
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HUMAN NEUROPEPTIDE Y-Y1 RECEPTORField of the Invention

The present invention relates to cDNA and genomic DNA sequences which encode the human neuropeptide Y-Y1
5 receptor. In addition the present invention relates to the use of these sequences in the production of the human neuropeptide Y-Y1 receptor and related receptor subtypes using recombinant DNA technology and to methods of screening and testing compounds for neuropeptide Y (NPY)
10 agonist or antagonist activity.

Background of the Invention

Neuropeptide Y (NPY) has a wide range of functions in the body, particularly affecting the cardiovascular system. Within the peripheral nervous system NPY is
15 present in postganglionic sympathetic nerves, being co-localised and co-released with other neurotransmitter, including catecholamines. When used pharmacologically, NPY has been shown to have a potent vasoconstrictor activity as well as dramatically potentiating the
20 vasoconstriction caused by many other pressor agents. Particularly high concentrations of NPY are found in the sympathetic nerves supplying the coronary, cerebral and renal vasculature and when infused into these vascular beds, NPY causes prolonged vasoconstriction that is not
25 reversed by adrenergic blocking agents. These observations have led to the proposal that NPY is the candidate transmitter for pathological vasospasm, a major cause of morbidity and mortality when involving the coronary and cerebral vessels.

30 NPY also appears to be involved in interaction with the renin angiotensin system. NPY containing sympathetic nerve terminals are found on the juxta-glomerular apparatus of the renal cortex and NPY influences renin release. These data, together with the demonstration of
35 alterations in NPY concentrations in hypertensive animal

models and the pressor response to infusion of the peptide, have resulted in implications of this peptide in hypertension.

Within the central nervous system NPY is localised
5 predominantly within interneurons where it appears to have a regulatory role. It therefore has widespread and diverse effects including effects on memory and a possible role in Alzheimer's disease. NPY is the most potent known
10 substance to cause an increase in feeding and may play a role in the genetic basis of Type II diabetes mellitus. NPY may also play a role as a regulatory agent in pituitary function as well as potential neuromodulatory function in stress responses and in reproductive function.

Specific agonists and antagonists of NPY are
15 therefore likely to be of substantial benefit for therapy of a wide range of clinical disorders. As NPY possess a compact tertiary structure and different parts of the molecule are required for interaction with different subtypes of the receptor, the logical development of both
20 agonists and antagonists is critically dependent upon the availability and knowledge of specific receptor structure.

NPY binds specifically to at least two receptors, Y1 and Y2. (Fuhlendorff, J., et al., Proc. Natl. Acad. Sci. USA. 87:182-186, 1990). In addition, a third receptor
25 subtype has been suggested (Wahlstedt, et al, Life Sciences 50:PL7-PL12, 1991; Michel, MC Trends in Pharmacol. Sci. 12:389-394, 1991). While it has been demonstrated that NPY receptors couple to the adenylate cyclase second messenger system, it remains probable that
30 additional NPY receptor subtypes exist since there is evidence that phosphatidylinositol turnover, cations, and arachidonic acid may also function as second messengers for NPY. Since NPY agonists and antagonists may have commercial value as potential anti-hypertensive agents,
35 cardiovascular drugs, neuronal growth factors,

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anti-psychotics, anti-obesity and anti-diabetic agents, the ability to produce NPY receptors by recombinant DNA technology would be advantageous.

The present inventors have isolated full length cDNA clones encoding the human Y1 NPY receptor (designated Y1) from human hippocampal cDNA using DNA homology screening. The receptor sequences were identified as the human Y1 NPY receptor by expression of the cloned cDNA in mammalian cells and by measurement of specific binding to the transfected cells by a variety of NPY analogues. The receptor has also been shown to couple to both the inhibition of adenylate cyclase activity and increases in intracellular cytosolic calcium levels. In addition, the receptor has been expressed in a bacterial cells, allowing for additional drug screening methods as well as purification of the receptor protein. The DNA sequences represents a novel human receptor which may be of clinical and commercial importance.

Summary of the Invention

Accordingly, in a first aspect the present invention consists in a cDNA molecule encoding the human NPY-Y1 receptor, the cDNA molecule having a sequence substantial as shown in Table 1 or a functionally equivalent sequence.

In a second aspect the present invention consists in a genomic DNA molecule encoding the human NPY-Y1 receptor, the genomic DNA molecule having a sequence substantially as shown in Table 2 or a functionally equivalent sequence.

As used herein the term "functionally equivalent sequence" is intended to cover minor variation in the DNA sequence which, due to degeneracy in the DNA code, do not result in the sequence encoding a different polypeptide. Further, this term is intended to cover alterations in the DNA code which lead to changes in the encoded polypeptide, but in which such changes do not affect the biological activity of the peptide. In addition, this term is

intended to cover use of the human NPY Y1 receptor gene or gene fragments for expression in cell lines to be used in drug screening.

In a third aspect the present invention consists in a method of producing human NPY-Y1 receptors comprising culturing a cell transformed with the cDNA molecule of the first aspect of the present invention or the genomic DNA molecule of the second aspect of the present invention under conditions which allow expression of the DNA sequence and optionally recovering the human NPY-Y1 receptor.

Where the cDNA sequence is used the cells may be either mammalian cells or bacterial cells. Where the cells are mammalian cells it is presently preferred that the cells are Chinese Hamster Ovary (CHO) cells or human embryonic kidney 293 cells.

It will also be clear to persons skilled in the art that where the genomic DNA sequence is used that gene fragments could be used to obtain expression of the NPY-Y1 receptor. It is intended that the use of such gene fragments is included within the scope of the present invention.

In a further preferred embodiment the cDNA molecule is under the control of the CMV promoter when expressed in mammalian cells.

In a fourth aspect the present invention consists in a method of screening compounds for NPY agonist or antagonist activity, comprising contacting the molecule with the human NPY-Y1 receptor produced by the method of the second aspect of the present invention.

In a preferred embodiment of the present invention the NPY-Y1 receptor is present on the surface of a cell, preferably CHO or 293 cells or bacterial cells.

The cDNA and genomic DNA molecules of the present invention represent novel human receptors. These

receptors may be of interest both clinically and commercially as they are expressed in many regions of the body and a NPY affects a wide number of systems.

By using the cDNA or genomic DNA sequences of the present invention it is possible to isolate the neuropeptide Y-Y1 receptor protein in a substantially pure form.

Accordingly, in a fifth aspect the present invention consists in neuropeptide Y-Y1 receptor in a substantially pure form.

It is believed that sequence of the present invention will also enable the isolation of DNA sequences encoding other NPY-Y1 receptor subtypes including the NPY-Y2 and NPY-Y3 receptor, by using the proof in DNA homology screening of DNA libraries of interest.

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following examples and drawings in which:-

Figure 1. Inhibition of porcine [125 I]-PYY binding with various NPY-related peptides. Increasing concentrations of human NPY [\square], human [L^{31}, P^{34}]NPY [\blacksquare], porcine peptide YY [\blacktriangle], peptide YY residues 13-36 [\bullet], and human pancreatic polypeptide [Δ] were tested for their ability to inhibit the binding of [125 I]-PYY. Results were expressed as a percentage of the maximal specifically bound radiolabelled PYY. Standard errors of triplicate samples are shown. Untransfected CHO cells showed no specific binding of NPY.

Figure 2. The cyclic AMP response, in 293 cells transfected with human NPY Y1 receptor, to NPY (L^{31}, P^{34}) (Y1) or porcine PYY-(13-36) is expressed as a percentage of the cyclic AMP level produced with 10 micromolar forskolin from three independent experiments.

Basal levels were 2.5 ± 0.13 pmol per 10^6 cells (untransfected 293 cells) and 2.26 ± 0.06 pmol per 10^6 cells (transfected 293 cells). Cyclic AMP levels stimulated by 10 micromolar forskolin were 121.2 ± 19.8 pmol per 10^6 cells (untransfected 293 cells) and 139 ± 14.2 pmol per 10^6 cells (transfected 293 cells)..

Figure 3. Intracellular calcium levels of fura-2-loaded CHO cells transfected with the human NPY Y1 receptor cDNA were measured in response to NPY, NPY (L^{31},P^{34}) (Y1), PYY, PYY 13-36, or PP. After successive addition of two of these compounds the intracellular calcium response mediated by the endogenous bombesin receptor was measured with the addition of 1 micromolar bombesin (Bom). The NPY Y1 receptor expressing cell line was stimulated with 100 nM NPY(A), peptide YY (B), NPY (L,P) (C), peptide YY cells did not respond to any of the NPY analogues, including 100 nM NPY (L^{31},P^{34}) (F). Treatment of stably transfected cells overnight with pertussis toxin at 100 ng/ml abolished the response to 2.5 micromolar PYY (H), as compared with untreated cells (G), but did not affect the intracellular response to 1 micromolar bombesin. The intracellular calcium increase mediated by the NPY Y1 receptor was dependent on the concentration of NPY (L^{31},P^{34}) and was maximal at 10 nM (J).

Figure 4 shows the restriction map for SacI, XhoI, BamHI and EcoRI of λ C clone.

Figure 5 shows the results obtained when CHO cells expressing human NPY-Y1 receptor were loaded with FURA-2 AM and stimulated with 5nM or 50nM human NPY after the addition of 40 μ g/ml hexapeptide. Increases in calcium induced by the endogenous bombesin receptor were measured by the addition of 5 nM bombesin.

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Methods:

Isolation of cDNA

Total RNA (3 μ g) from rat brain was used as a template to synthesize random primed single-stranded cDNAs. These cDNAs were used in a polymerase chain reaction (PCR) together with the oligonucleotide primers R1 (CTG GTG CTG CAG TAT TTT GGC CCA CTC TGT) and R2 (AAT GTC TCA GAG AAT TCT CCA TTT CTG GCC) 30 pmol each) which correspond to position 672-584 and 48-78 in the rat cDNA clone FC5R, respectively. PCR condition: 30 cycles at 95 for 1 min, 63 C for 2 min and 72 C for 1 min. The reaction product was digested with EcoR I and Pst I, gel purified and subcloned for sequencing into the Bluescript vector (Stratagene) to show authenticity.

Two lambda cDNA libraries derived from human fetal brain (Clonetec) and human adult hippocampus (Stratagene) (9.10^5 pfu each) were screened with the rat cDNA as a probe under following hybridization conditions: 5 x SSPE, 0.1% SDS and 5x Denhardt at 60 C for 16 hours. The filters were washed twice with 2 x SSC and 0.1% SDS at 60 C for 15 min. Three strongly hybridizing clones were isolated and the cDNA inserts were subcloned for sequencing into Bluescript vectors. The largest cDNA (2.5 kb) contains an open reading frame for 384 amino acids encoding the human NPY receptor subtype Y₁ (Table 1). The two other clones (F5 and F13) are truncated versions of the same cDNA with 100% identity in the overlapping region (position 664-1555 and 670-1925) respectively.

The mammalian expression construct pN-H(3-4) was made by subcloning a PCR fragment containing only the coding region of the NPY Y₁ receptor into the pcDNA NEO vector. The construct is under the control of the CMV promoter and contains the neomycin gene for selection.

The expression construct pN-H3 was transfected into the mammalian cell line CHO K1 using a modified calcium

TABLE 1/1

Human Neuropeptide Y Y1 receptor cDNA

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70
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ATTGTTCAAGGGAATGAAGAATTCAGAATAATTTGGTAAATGGATTCCAATATCGGGAATAAGA
140
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*
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*
*
ATAAGCTGAACAGTTGACCTGCTTTGAAGAAACATACTGTCCATTGTCTAAAATAATCTATAACAACCA
210
*
*
*
*
*
AACCAATCAAAATGAATTCAACATTATTTTCCCAGGTTGAAAATCATTTCAGTCCACTCTAATTTCTCAGA
M N S T L F S Q V E N H S V H S N F S E
280
*
*
*
*
*
GAAGAATGCCAGCTTCTGGCTTTTGAAGATGATGATTGTCATCTGCCCTTGGCCATGATATTTACCTTA
K N A Q L L A F E N D D C H L P L A M I F T L
350
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*
*
GCTCTTGCTTATGGAGCTGTGATCATTCTTGGTGTCTCTGGAAACCTGGCCTTGATCATAATCATCTTGA
A L A Y G A V I I L G V S G N L A L I I I I L
420
*
*
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*
*
AACAAAAGGAGATGAGAAATGTTACCAACATCCTGATTGTGAACCTTTCCTTCTCAGACTTGCTTGTTC
K Q K E M R N V T N I L I V N L S F S D L L V A
490
*
*
*
*
*
CATCATGTGTCTCCCTTTACATTTGTCTACACATTAATGGACCACTGGGTCTTTGGTGAGGCGATGTGT
I M C L P F T F V Y T L M D H W V F G E A M C
560
*
*
*
*
*
AAGTTGAATCCTTTTGTGCAATGTGTTTCAATCACTGTGTCCATTTTCTCTCTGGTTCTCATTGCTGTGG
K L N P F V Q C V S I T V S I F S L V L I A V
630
*
*
*
*
*
AACGACATCAGCTGATAATCAACCCTCGAGGGTGGAGACCAATAATAGACATGCTTATGTAGGTATTGC
E R H Q L I I N P R G W R P N N R H A Y V G I A
700
*
*
*
*
*
TGTGATTGGGTCCTTGCTGTGGCTTCTTCTTTGCCTTTCCTGATCTACCAAGTAATGACTGATGAGCCG
V I W V L A V A S S L P F L I Y Q V M T D E P
770
*
*
*
*
*
TTCCAAAATGTAACACTTGATGCGTACAAAGACAAATACGTGTGCTTTGATCAATTTCCATCGGACTCTC
F Q N V T L D A Y K D K Y V C F D Q F P S D S
840
*
*
*
*
*
ATAGGTTGTCTTATACCACTCTCCTCTTGGTGCTGCAGTATTTTGGTCCACTTTGTTTTATATTTATTG
H R L S Y T T L L L V L Q Y F G P L C F I F I C
910
*
*
*
*
*
CTACTTCAAGATATATATACGCCTAAAAAGGAGAAACAACATGATGGACAAGATGAGAGACAATAAGTAC
Y F K I Y I R L K R R N N M M D K M R D N K Y
980
*
*
*
*
*
AGGTCCAGTGAAACCAAAAGAATCAATATCATGCTGCTCTCCATTGTGGTAGCATTGTCAGTCTGCTGGC
R S S E T K R I N I M L L S I V V A F A V C W
1050
*
*
*
*
*
TCCCTCTTACCATCTTTAACACTGTGTTTGATTGGAATCATCAGATCATTGCTACCTGCAACCACAATCT
L P L T I F N T V F D W N H Q I I A T C N H N L

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TABLE 1/2

* * * * * 1120
 GTTATTCCTGCTCTGCCACCTCACAGCAATGATATCCACTTGTGTCAACCCCATATTTTATGGGTTCCTG
 L F L L C H L T A M I S T C V N P I F Y G F L
 * * * * * 1190
 AACAAAACTTCCAGAGAGACTTGCAGTTCTTCTTCAACTTTTGTGATTTCCGGTCTCGGGATGATGATT
 N K N F Q R D L Q F F F N F C D F R S R D D D
 * * * * * 1260
 ATGAAACAATAGCCATGTCCACGATGCACACAGATGTTTCCAAACTTCTTTGAAGCAAGCAAGCCCAGT
 Y E T I A M S T M H T D V S K T S L K Q A S P V
 * * * * * 1330
 CGCATTTAAAAAATCAACAACAATGATGATAATGAAAAATCTGAAACTACTTATAGCCTATGGTCCCG
 A F K K I N N N D D N E K I *
 * * * * * 1400
 GATGACATCTGTTTAAAAACAAGCACACCTGCAACATACTTTGATTACCTGTTCTCCCAAGGAATGGGG
 * * * * * 1470
 TTGAAATCATTTGAAAATGACTAAGATTTTCTTGTCTTGCTTTTTACTGCTTTTGTTGTAGTTGTCATAA
 * * * * * 1540
 TTACATTTGGAACAAAAGGTGTGGGCTTTGGGGTCTTCTGGAAATAGTTTTGACCAGACATCTTTGAAGT
 * * * * * 1610
 GCTTTTTGTGAATTTATGCATATAATATAAAGACTTTTATACTGTACTTATTGGAATGAAATTTCTTTAA
 * * * * * 1680
 AGTATTACGATNNNCTGACTTCAGAAGTACCTGCCATCCAATACGGTCATTAGATTGGGTCATCTTGATT
 * * * * * 1750
 AGATTAGATTAGATTAGATTGTCAACAGATTGGGCCATCCTTACTTTATGATAGGCATCATTTTAGTGTG
 * * * * * 1820
 TTACAATAGTAACAGTATGCAAAAGCAGCATTTCAGGAGCCGAAAGATAGTCTTGAAGTCATTTCAGAAGTG
 * * * * * 1890
 GTTTGAGGTTTCTGTTTTTTGGTGGTTTTTGTGTTTTTTTTTTTTTTTTCACCTTAAGGGAGGCTTTCAT
 * * * * * 1960
 TTCCTCCCGACTGATTGTCACTTAAATCAAATTTAAAAATGAATAAAAAGACATACTTCTCAGCTGCAA
 * * * * * 2030
 ATATTATGGAGAATTGGGCACCCACAGGAATGAAGAGAGAAAGCAGCTCCCCAACTTCAAACCATTTTG
 * * * * * 2100
 GTACCTGACAACAAGAGCATTTTAGAGTAATTAATTTAATAAAGTAAATTAGTATTGCTGCAAATAGCTA
 * * * * * 2170
 AATTATATTTATTTGAATTGATGGTCAAGAGATTTTCCATTTTTTTTACAGACTGTTCAAGTGTGTTGTCAA
 * * * * * 2240
 GCTTCTGGTCTAATATGTACTCGAAAGACTTTCCGCTTACAATTTGTAGAAACACAAATATCGTTTTCCA
 * * * * * 2310
 TACAGCAGTGCCTATATAGTACTGATTTTAACTTTCAATGTCCATCTTTCAAAGGAAGTAACACCAAGG
 * * * * * 2380
 TACAATGTTAAAGGAATATTCACTTTACCTAGCAGGGAAAAATACACAAAACTGCAGATACTTCATATA

-10-

TABLE 1/3

2450

* * * * *
GCCCATTTTAACTTGTATAAACTGTGTGACTTGTGGCGTCTTATAAATAATGCACTGTAAAGATTACTGA
2520

* * * * *
ATAGTTGTGTCATGTTAATGTGCCTAATTTTCATGTATCTTGTAATCATGATTGAGCCTCAGAATCATTG
2590

* * * * *
GAGAACTATATTTTAAAGAACAAGACATACTTCAATGTATTATACAGATAAAGTATTACATGTGTTGA

* * * * *
TTTTAAAAGGGCGGACATTTTATTAAAATCAAGG

SUBSTITUTE SHEET

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phosphate transfection method. Stably transfected cells were selected with neomycin and tested for the ability to bind NPY/PYY peptide analogues. Transfected cells (1×10^6) were incubated in 0.5ml assay buffer (50mM Tris-HCl pH 7.4, 2mM CaCl_2 , 5mM KCl, 120mM NaCl, 1mM MgCl_2 , and 0.1% bovine serum albumin) in the presence of 0.05nM [^{125}I]-labelled peptide YY(NEN) and either 10^{-8}M porcine peptide YY(PYY 13-36), or the Y1 receptor-selective NPY(Leu31,Pro34)[peptides were obtained from Auspep, Australia]. Peptide analogues were also tested on human neuroblastoma SK-N-MC cells (1.6×10^6), which express only the Y1 receptor subtype, and on rat brain membrane preparations (approximately 100 μg) which express predominantly Y2 receptors. Untransfected CHO K1 cells (1×10^6) were also tested and showed no specific binding to the peptide analogues. Cells were incubated with the radiolabelled peptide and unlabelled competitors for one hour at room temperature and pelleted in a microcentrifuge for 4 minutes. Pellets were counted for one minute in a gamma counter. Binding of the Y1 receptor-selective agonist confirmed the identity of this clone as the NPY-Y1 receptor.

The expressed NPY Y1 receptor was assessed for its ability to bind NPY and related analogs (methods detailed in human NPY Y1 receptor patent application) (Figure 1). The receptor has been successfully used to show specific binding to a Y1 receptor-specific agonist NPY ($\text{L}^{31}, \text{P}^{34}$) and to not bind to a Y2 receptor-specific agonist peptide YY 13-36. These results reflect the in vivo effects of these agonists.

In the present invention, the Y1 receptor expressed in these cell lines has been shown to couple to inhibition of adenylate cyclase in 293 cells (Figure 2) and the increase in cytosolic calcium levels in the CHO cells (Figure 3). These are significant and novel findings and

-12-

of use in the screening of agonists and antagonists by function.

To measure intracellular cyclic AMP levels, cyclic AMP was assayed in whole cells treated for 15 min. at 37°C with 100 micromolar isobutylmethylxanthine (IBMX; Sigma). Transfected cells (1×10^6 / 0.5 ml reaction) were incubated with 10 micromolar forskolin and various concentrations of NPY and related peptides. Reactions were terminated with the addition of HCl to 0.1M, incubation at room temperature for 15 min., neutralisation and sample dilution in 50 mM sodium acetate, pH 6.2. Cyclic AMP was quantitated by using a radioimmunoassay (Dupont/NEN).

To measure levels of intracellular calcium, transfected cells were suspended in loading medium (modified RPMI 1640 medium/10 mM Hepes/1% newborn calf serum) and incubated in a spinner flask at 37°C for 2.5 hour at 1×10^6 cells per ml. Cells were then treated with 1 micromolar Fura-2 acetoxymethyl ester (fura-2 AM; Molecular Probes) for 30 min. at 37°C, washed twice with loading medium, and resuspended at 5×10^6 cells/ml. Immediately before fluorescence spectroscopy, cells were recovered by centrifugation at 1000 rpm and resuspended at 1×10^6 cells/ml in a modified Krebs buffer (135 mM NaCl/4.7 mM KCl/1.2 mM MgSO_4 /1.2 mM KH_2PO_4 /5 mM NaHCO_3 /1 mM CaCl_2 /2.8 mM glucose/10 mM Hepes, pH 7.4) containing sulfinpyrazone. Bombesin was purchased from Sigma and Auspep. Fluorescence recordings were made on a Hitachi fluorescence spectrometer (F4010) at 340 nm (excitation) and 505 nm (emission) over 10 min. with slit widths of 5 nm and response time of 2 seconds. Intracellular calcium was quantitated by using equations described by Grynkiewicz, et al., J. Bio. Chem. 260:3440-3450, 1985.

SUBSTITUTE SHEET

Plasmid Construction

Plasmid pMalp (Promega), which carries the male gene under the control of the tac promoter, was cut with EcoRI and HindIII restriction enzymes simultaneously. A 2.1kb
5 EcoRI/HindIII cDNA fragment containing the whole coding region for the human NPY Y1 receptor was cloned into the pMalp vector to generate plasmid pHz59. The plasmid pHz59 was cut with StuI and EcoRI restriction enzymes simultaneously and the 5' overhang of the EcoRI site was
10 filled in with Klenow enzyme and the plasmid was religated to generate the fusion construct pHz60. The recombinant plasmid (pHz60) was transfected into the bacterial strain TB1.

Preparation and Fractionation of E.coli Membranes

15 Bacteria were grown at 37°C in L broth containing 50lg/ml of ampicillin, up to an A600 value of 0.5-0.7. Derepression of the tac promoter with 1mM isopropylb-D-thiogalactopyranoside (IPTG) for 2 hours at 37 C let to the production of a 86 kD male/NPY Y1 receptor
20 fusion-protein. Cells, from a 1l culture, were harvested and washed at 4°C with 1l 10mM Hepes pH 7.5 and the pellet stored over night at -20°C. All the subsequent steps were carried out on ice. The bacterial pellet was suspended in 28 ml 10mM Hepes pH 7.5 containing 20% (by
25 mass) sucrose, DNase I at 30 lg/ml, and RNase A at 30 lg/ml, and the following protease inhibitors: 1mM phenylmethylsulfonylfluoride, leupeptin at 5 lg/ml, and pepstatin at 7 lg/ml. The suspension was sonicated three times for 3 min. Unbroken cells were removed by
30 centrifugation at 5000g for 10 min. and 2 ml 0.1 M EDTA (pH 7.5) was added to the supernatant. This supernatant was layered on a sucrose gradient consisting of 3ml 60% (mass/vol.) sucrose, 6 ml 42.5% (mass/vol.) sucrose, and 15 ml 25% (mass/vol.) sucrose (in 10 mM Hepes pH7.5
35 containing 5 mM EDTA) and centrifuged at 100000g for 16h

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at 4°C in a Beckmann SW41 rotor. Two major bands were visible at the interface of the sucrose layers, corresponding to the enriched preparations of the outer and inner membrane. Fractions were collected from the gradient and aliquots used to measure binding activity for ¹²⁵I NPY, in the binding assay described.

Isolation of the Human NPY Y1 Receptor Gene

A human genomic DNA library constructed in lambda GEM 11 phage vector (Clontec) was screened with a ³²P-labelled cloned NPY Y1 receptor cDNA (nucleotides 14 to 2327) isolated from human hippocampal cDNA. Two positive clones were obtained from 7.5x10⁵ bacteriophage plaques. Clone kC contained an insert of approximately 14 kb and clone kD contained an insert of approximately 11kb. The insert in kD was subsequently shown to be completely contained within the longer clone. The restriction map for Sac I, Xho I, BamH I and EcoR I of the kC clone is shown in Fig. 4 and sequence set out in Table 2. The insert of this clone was digested with different enzymes and the fragments subcloned into the Bluescript SK vector for sequencing. The exon sequence of the NPY Y1 receptor gene is identical to that of the human hippocampal cDNA described above. The human NPY Y1 receptor gene consists of 3 exons. This is in contrast to many of the other G protein coupled receptor genes, which are intronless. The overall sequence of the gene consists of approximately 10kb (Fig. 4). The first 57 nucleotides of the 5' untranslated sequence of the human hippocampal NPY Y1 receptor mRNA are separated by a 6kb intron from the second exon. The second intron (97bp), containing an in frame stop codon, is located exactly after the proposed fifth transmembrane domain at nucleotide 908 corresponding to the cDNA sequence (Table 2). Introns in several other G-coupled receptor genes (human substance K, human rhodopsin), tend to be positioned the same way, shortly

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TABLE 2/1

* * * * *
 AACGTACTCGTGACATTCTATTTTTTCTTCATAATGTTCACTACTGTAGTACTAATCACCAGAGAAAAT
 * * * * *
 TGCATTGACTCTTTTCGACCACCAGGGAAATATTCAGCTCATGGTTCTCCCCAAAAAACTAAAAAGCAG
 * * * * *
 CTAAGCGCTGGGAACAAATCTGACTTATTGCATTTTCTCAGTGGGCCAAAGAAAGGAGGGCCGATTGACT
 * * * * *
 GCTTTGACTTTTTAAAGGTCTTCTCTTTGTTCACTTATAAAGTGAGGAAAACAAATTCTCGGCACTGGCG
 * * * * *
 TGAGAGTTGAGCGTCACAAAAGAAAGCAAAAGAAAATATTAGTGCCATTATTGTGGCGAATTTTCATGTTT
 * * * * *
 AP2
 CCCAGCGAGCCCTTTGATTCTGTTTGGGCTGGCGCTCGAGCTCTCCAGCCGGGTATGACTTCGGCCAC
 * * * * *
 AAGATGGCACTGACCTGCAAACAAAGAAAAGCACAGTGGCACCAGCTTTTCAAGCCTCGGGAAACTGCC
 * * * * *
 CTGCCTTCCCGGAGTCGAGGACTGTGGGGATTAGGGCTTCTTCCCTGCGCGGGAGGTCTGTGTGCA
 * * * * *
 ATAATGTGTGGCTTCTGTTGGATTGCTTTTCTTCCAAAATTCCTAGGCAATGCTTCCCGAGGTGTGCA
 * * * * *
 CRE
 CCTTTGTGAGGTGTTTGTGGGGTTGGGGGAGCTTCAGGCGCTACTCGCGGGACGACGTACGTGATCCGG
 * * * * *
 CAAT
 GATGAGGTGGAGTTCGGCTTTAAGGAGGCGTCTCTTCTAGCTTCAATCAATCTTTAGGATCTGAGCAGGA
 * * * * *
 TATA
 GAAATAACAGCGGATCTTCCCACTCTGCTCCCTTCCATTCCACCCCTTCTTTAATAAGCAGGAGC
 * * * * *
 +1
 GAAAAGACAAATTCCAAAGAGGTAAGTTGCGAGTTTATGCCTTTCAGAGACTTCTGCGAAATCTCTC
 * * * * *
 +57
 ATTGACAAGGTGAAGGATGAGAGGGGAAGAAAACGATGCGAGTSTCCGAAACTGGCTCTGGGGGACCAA
 * * * * *
 GGTGGGGTCTCCAGTGCAGGCAGGTGCGAGGTTGCTCCAAATCACCAGGACCGTTCGCGGGCTCCTGCCGA
 * * * * *
 AGGGTATGGGGACGACGCGGGTGAAAGGAGAGGGTACCCTACGCGGAGTTCGGGCTTTCCCCCACCTGC
 * * * * *
 TCCCGGGGAATTTCTGGATGGGGATCCAAGTTTCTTTACCCCGTTCTCTTTAAAGGCCGAGGCACT
 * * * * *
 GGGAAGGCGCCCGAGTTCGCCTATCCACACCCGCTCATTTTCTTACGTGTCTTGAGCTGGCTGGAGGC
 * * * * *
 ACTGGCTCTGGCCGCACCGAGTTTTCGCGGAGTAACTGGCTGGGATGAGCCGGAGAAGGGTGGGCTTGC
 * * * * *
 ACGTCGCCCAGCGTCGCACGGGTCCGCGGCAGGTGTCGGGCTGGGAGCTGGCGGAGGTGGGAGTAGCCCT
 * * * * *
 CGGCGCGGACCCAGCGCGCAAGCCCACCCCACTTGCGGGTTCCGCGCTTCTCGGCTGCAATCGAGCCG

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TABLE 2/2

* * * * *
 CGCACCGCAGTACCGGGCGCTTCGAGTGTGGCGCTGCGCCCCGATAGACACCCGAAGCTTTTAATCATCG
 * * * * *
 GAGTTCTAATCAGGGTTCTCTTTAGCTCTTTCTTTTAGAAAAGTAATGATGGAACGTGGCTGG-----
 ----- 6 kb -----
 * * * * *
 -----TATTCCTTACTAACTTATAGACCATTATGTTTACTAAGACTTGTCTGTAAGCAAGTCC
 * * * * *
 AATTAAAAATTTTATTTTTTCTTTATTTCAGTATGTTTTACCATTCTGCTATTTTAGAAAGATGTTTAC
 * * * * *
 AAGATTACATTTTGTATTATTTCAGTGTTTTCACTTTAAAGAGTTCTGTGAGTCAGAAGTCATTTTG
 * * * * *
 ACTGCCCTCAATAAAATTAGTAATGCAATTGGTCATTTTCTCTTACAGATTGTTTCAGTTCAAGGGAATG
 * * * * * +58
 AAGAATTCAGAATAATTTTGGTAAATGGATTCCAATATCGGGAATAAGAATAAGCTGAACAGTTGACCTG
 * * * * *
 CTTTGAAGAAACATACTGTCCATTTGTCTAAAATAATCTATAACAACCAACCAATCAAAATGAATTCAA
 * * * * * M N S
 * * * * *
 CATTATTTTCCCAGGTTGAAAATCATTTCAGTCCACTCTAATTTCTCAGAGAAGAATGCCAGCTTCTGGC
 * * * * *
 T L F S Q V E N H S V H S N F S E K N A Q L L A
 * * * * *
 TTTTGAAAATGATGATTGTCATCTGCCCTTGGCCATGATATTTACCTTAGCTCTTGCTTATGGAGCTGTG
 * * * * *
 F E N D D C H L P L A M I F T L A L A Y G A V
 * * * * *
 ATCATTCTTGGTGTCTCTGGAACCTGGCCTTGATCATAATCATCTTGAAACAAAAGGAGATGAGAAATG
 * * * * *
 I I L G V S G N L A L I I I I L K Q K E M R N
 * * * * *
 TTACCAACATCCTGATTGTGAACCTTTCCTTCTCAGACTTGCTTGTTGCCATCATGTGTCTCCCCTTTAC
 * * * * *
 V T N I L I V N L S F S D L L V A I M C L P F T
 * * * * *
 ATTTGTCTACACATTAATGGACCACTGGGTCTTTGGTGAGGCGATGTGTAAGTTGAATCCTTTTGTGCAA
 * * * * *
 F V Y T L M D H W V F G E A M C K L N P F V Q
 * * * * *
 TGTGTTTCAATCACTGTGTCCATTTTCTCTCTGGTTCTCATTGCTGTGGAACGACATCAGCTGATAATCA
 * * * * *
 C V S I T V S I F S L V L I A V E R H Q L I I
 * * * * *
 ACCCTCGAGGGTGGAGACCAAATAATAGACATGCTTATGTAGGTATTGCTGTGATTGCGGTCTTGCTGT
 * * * * *
 N P R G W R P N N R H A Y V G I A V I W V L A V
 * * * * *
 GGCTTCTTCTTTGCCTTTCCTGATCTACCAAGTAATGACTGATGAGCCGTTCCAAAATGTAACTTGTAT
 * * * * *
 A S S L P F L I Y Q V M T D E P F Q N V T L D
 * * * * *
 GCGTACAAAGACAAATACGTGTGCTTTGATCAATTTCCATCGGACTCTCATAGGTTGTCTTATACCACTC
 * * * * *
 A Y K D K Y V C F D Q F P S D S H R L S Y T T

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TABLE 2/3

* * * * * +908 *
 TCCTCTTGGTGCTGCAGTATTTTGGTCCACTTTGTTTATATTTATTTGCTACTTCAAGTAAGAAACT
 L L L V L Q Y F G P L C F I F I C Y F K

* * * * *
 TTTTCTATCATTCCATTTTACCTTCTTTACACAGAATTCCTCATCAAATGAGTGTTCATTAA

* * +909* * * * *
 CTTTTTCTTCCATAGATATATATACGCCTAAAAAGGAGAAACAACATGATGGACAAGATGAGAGACAAT
 I Y I R L K R R N N M M D K M R D N

* * * * *
 AAGTACAGGTCCAGTGAAACAAAAGAATCAATATCATGCTGCTCTCCATTGTGGTAGCATTGTCAGTCT
 K Y R S S E T K R I N I M L L S I V V A F A V

* * * * *
 GCTGGCTCCCTCTTACCATCTTTAACTGTGTTTGATTGGAATCATCAGATCATTGCTACCTGCAACCA
 C W L P L T I F N T V F D W N H Q I I A T C N H

* * * * *
 CAATCTGTTATTCCTGCTCTGCCACCTCACAGCAATGATATCCACTTGTGTCAACCCCATATTTTATGGG
 N L L F L L C H L T A M I S T C V N P I F Y G

* * * * *
 TTCCTGAACAAAACCTTCCAGAGAGACTTGCAGTTCCTTCTCAACTTTTGTGATTTCCGGTCTCGGGATG
 F L N K N F Q R D L Q F F F N F C D F R S R D

* * * * *
 ATGATTATGAAACAATAGCCATGTCCACGATGCACACAGATGTTTCCAAAACCTCTTTGAAGCAAGCAAG
 D D Y E T I A M S T M H T D V S K T S L K Q A S

* * * * *
 CCCAGTCGCATTTAAAAAATCAACAACAATGATGATAATGAAAAATCTGAAACTACTTATAGCCTATG
 P V A F K K I N N N D D N E K I

* * * * *
 GTCCCGGATGACATCTGTTTAAAAACAAGCACACCTGCAACATACTTTGATTACCTGTTCTCCCAAGGA

* * * * *
 ATGGGGTTGAAATCATTGAAATGACTAAGATTTTCTTGTCTTGCTTTTTACTGCTTTTGTGTAGTTG

* * * * *
 TCATAATTACATTTGGAACAAAAGGTGTGGGCTTTGGGGTCTTCTGGAAATAGTTTGGACCAGACATCTT

* * * * *
 TGAAGTGCTTTTGTGAATTTATGCATATAATATAAAGACTTTTATACTGTACTTATTGGAATGAAATTT

* * * * *
 CTTTAAAGTATTACGATNNCTGACTTCAGAAGTACCTGCCATCCAATACGGTCATTAGATTGGGTCATC

* * * * *
 TTGATTAGATTAGATTAGATTAGATTGTCAACAGATTGGGCCATCCTTACTTTATGATAGGCATCATTTT

* * * * *
 AGTGTGTTACAATAGTAACAGTATGCAAAAGCAGCATTGAGGAGCCGAAAGATAGTCTTGAAGTCATTCA

* * * * *
 GAAGTGGTTTGAGGTTTCTGTTTTTGGTGGTTTTTGTGTTTTTTTTTTTTTTTACCTTAAGGGAGGC

* * * * *
 TTTCAATTCCTCCCGACTGATTGTCACTTAAATCAAATTTAAAAATGAATAAAAAGACATACTTCTCAG

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TABLE 2/4

* * * * *

CTGCAAATATTATGGAGAATTGGGCACCCACAGGAATGAAGAGAGAAAGCAGCTCCCCAACTTCAAACC

* * * * *

ATTTTGGTACCTGACAACAAGAGCATTTTAGAGTAATTAATTTAATAAAGTAAATTAGTATTGCTGCAAA

* * * * *

TAGCTAAATTATATTTATTTGAATTGATGGTCAAGAGATTTTCCATTTTTTTTACAGACTGTTTCAAGTGT

* * * * *

TGTCAAGCTTCTGGTCTAATATGTACTCGAAAGACTTTCCGCTTACAATTTGTAGAAACACAAATATCGT

* * * * *

TTTCCATACAGCAGTGCCTATATAGTGACTGATTTTAACTTAATGTCCATCTTTCAAAGGAAGTAACA

* * * * *

CCAAGGTACAATGTTAAAGGAATATTCACCTTTACCTAGCAGGGAAAAATACACAAAACACTGCAGATACTT

* * * * *

CATATAGCCCATTTTAACTTGTATAAACTGTGTGACTTGTGGCGTCTTATAAATAATGCACTGTAAAGAT

* * * * *

TACTGAATAGTTGTGTCATGTTAATGTGCCTAATTTTCATGTATCTTGTAAATCATGATTGAGCCTCAGAAT

* * * * *

CATTTGGAGAACTATATTTTAAAGAACAAGACATACTTCAATGTATTATACAGATAAAGTATTACATGT

* * * * *

GTTTGATTTTAAAGGGCGGACATTTTATTAATCAAGG

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after or in front of a transmembrane domain. This organisation is also consistent with suggestions that the following third cytoplasmatic loop of the receptor forms a specific domain involved in determination of the specificity of coupling to different G proteins. The nucleotide sequences of the two introns adjoining the splice junctions (Table 2) are consistent with the recognised consensus sequence GT/AG.

Putative regulatory sequence elements

Identification of the transcriptional initiation site was carried out with primer extension, using a 21mer primer corresponding to nucleotides 38 to 18. The primer when extended on mRNA derived from the NPY Y1 receptor specific expressing human neuroblastoma cell line SK-N-MC, revealed a transcription start site at 210 nucleotides upstream from the initiation start codon. This position is also identical with a type 1 cap site (CCATTC) and is accompanied 35bp upstream by a TATA box-like motif (AAATAC), a typical CAAT box (TCAATCT) 60bp upstream, a cAMP response element (CGACGTCA) 124 bp upstream and a AP2 recognition site (GCGAGCCC) 451bp upstream (Table 2). No other typical transcription factor binding sites are found in the + orientation in this region. A potential polyadenylation site (ATTAAA) was found at position 2670 of the cDNA sequence. The approximate 2.7 kb sequence of the cDNA is consistent with the size of the NPY Y1 receptor mRNA demonstrated in Northern analysis of placental and kidney mRNA.

Characterisation of a Pst I Polymorphism in the Human NPY

Y1 Receptor Gene

Southern blot analysis was performed using the ³²P-labelled cDNA fragment (nucleotides 14 to 2327) as a probe. The restriction patterns obtained with BamH I, EcoR I and Sac I correspond exactly to the fragments found in the human genomic clones. However, the pattern for the

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restriction enzyme Pst I shows an additional 5.1 kb band, which suggests the presence of a polymorphic site within the first intron of the gene. Southern blot analysis of genomic DNA samples from 69 normal individuals confirmed this suggestion and demonstrated that the allele frequency for the Pst I polymorphism in this population is 54%:46% (Table 3). Sequence analysis revealed a single point mutation in the recognition site for the restriction enzyme Pst I, changing the first cytidine to a thymidine. The polymorphism could be used to assess variations in the population which may represent a possible marker for hypertension.

Genomic Library Screening

A human peripheral blood genomic DNA library (Clontec) was screened with a ^{32}P -labelled 2.3kb fragment (nucleotides 14 to 2327) of the human NPY Y1 receptor cDNA. Bacteriophage DNA was transferred to Hybond N⁺ filters (Amersham) and hybridised with the probe in a solution containing 6xSSC, 5xDenhardt's and 0.1% SDS at 65°C for 16 h. Filters were washed twice 15 min in 2xSSC/0.1%SDS at 65°C followed by a 15 min wash in 0.1xSSC/0.1% SDS and exposed to X-ray film (Kodak, X-Omat) using an intensifying screen at -70°C for 16h. Positive plaques were purified and DNA was isolated using a standard lysate procedure. kDNA was digested with EcoR I, Hind III, BamH I, Sac I, Xho I, or combinations of these enzymes to generate subsequently ordered subclones in the Bluescript SK vector (Stratagene) covering the entire region of the NPY Y1 receptor gene.

Nucleotide Sequence Determination

Supercoiled plasmid DNA was alkaline-denatured and sequenced by the dideoxy chain termination method using T7 polymerase (Promega). Oligonucleotide primers were initially from the flanking region of the vector and internal to the cDNA sequence. Additional primers were

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synthesised based on intron sequence obtained.

TABLE 3: NPY Y1 Receptor Gene Pst I Polymorphism

5		Genotypes			Allele Frequency	
		3.8/3.8	3.8/5.1	5.1/5.1	3.8	5.1
	Samples (69)	22	30	17	74	64
10	Frequency	31.9%	43.4%	24.6%	53.6%	46.4%

NOY Agonist and Antagonist Sequencing

The mammalian cells transfected with the human NPY Y1 receptor cDNA were used to identify a peptide antagonist of NPY function.

Using synthetic peptide chemistry, a hexapeptide amide SALRHY-NH₂ (Ser-Ala-Leu-Arg-His-Tyr amide), which corresponds to residues 22-27 of the NPY molecule portion of the amphiphatic helix in NPY was synthesised in an ABI Peptide Synthesiser Model 430A. T-boc chemistry was used. HF cleavage was used to release peptide from the solid support. Peptide was subjected to HPLC (Ion Exchange and Reverse Phase) purifications. The peptide was subjected to sequence and amino acid analysis for sequence integrity. In a series of experiments not described here in anaesthetized rats significant inhibition of the NPY-evoked pressor response and a decrease in resting blood pressure levels 60 minutes after administration of SALRHY-NH₂ was observed. The inhibitory effect of SALRHY-NH₂ is confined to the postsynaptic or Y1 receptor as no significant inhibitory effects are seen on attenuation of cardiac vagal action, a Y2 receptor mediated function.

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The hexapeptide was tested in vitro for its ability to inhibit NPY effects on mammalian cell lines transfected with the human NPY Y1 receptor cDNA. The ability of the hexapeptide to block the NPY-induced increase in intracellular calcium correlated well with its in vivo ability to block NPY Y1 receptor-mediated increases in blood pressure.

CHO cells expressing the human NPY Y1 receptor were loaded with FURA-2 AM as described, and stimulated with 5 nM or 50 nM human NPY (Auspep) after the addition of 401g/ml SALRHY-NH2 hexapeptide. Increases in calcium induced by the endogenous bombesin receptor were measured by the addition of 5nM bombesin. The results are shown in Figure 5.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS:-

1. A cDNA molecule encoding the human NPY-Y1 receptor, the cDNA molecule having a sequence substantial as shown in Table 1 or a functionally equivalent sequence.
- 5 2. A genomic DNA molecule encoding the human NPY-Y1 receptor, the genomic DNA molecule having a sequence substantially as shown in Table 2 or a functionally equivalent sequence.
3. A method of producing human NPY-Y1 receptors
- 10 comprising culturing a cell transformed with the cDNA molecule as claimed in claim 1 or genomic DNA molecule as claimed in claim 2 under conditions which allow expression of the DNA sequence and optionally recovering the human NPY-Y1 receptor.
- 15 4. A method as claimed in claim 3 in which the method comprises culturing a bacterial cell transformed with the cDNA molecules as claimed in claim 1.
5. A method as claimed in claim 3 in which the cell is a mammalian cell.
- 20 6. A method as claimed in claim 5 in which the cells are Chinese Hamster Ovary cells or human embryonic kidney 293 cells.
7. A method as claimed in claim 5 or 6 in which the cell is transformed with a fragment of the genomic DNA molecule
- 25 as claimed in claim 2, the fragment including the coding regions for the NPY-Y1 receptor.
8. A method as claimed in any one of claims 3 to 7 in which the cDNA molecule is under the control of the CMV promoter.
- 30 9. A method of screening compounds for NPY agonist or antagonist activity, comprising contacting the molecule with the human NPY-Y1 receptor produced by the method as claimed in any one of claims 3 to 8.
10. A method as claimed in claim 9 in which the NPY1
- 35 receptor is present on the surface of a cell.

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11. A method as claimed in claim 10 in which the cell is a mammalian or bacterial cell.
12. A method as claimed in claim 11 in which the cell is Chinese Hamster Ovary cell or human embryonic kidney 293
- 5 cells.
13. . Neuropeptide Y-Y1 receptor in a substantially pure form.

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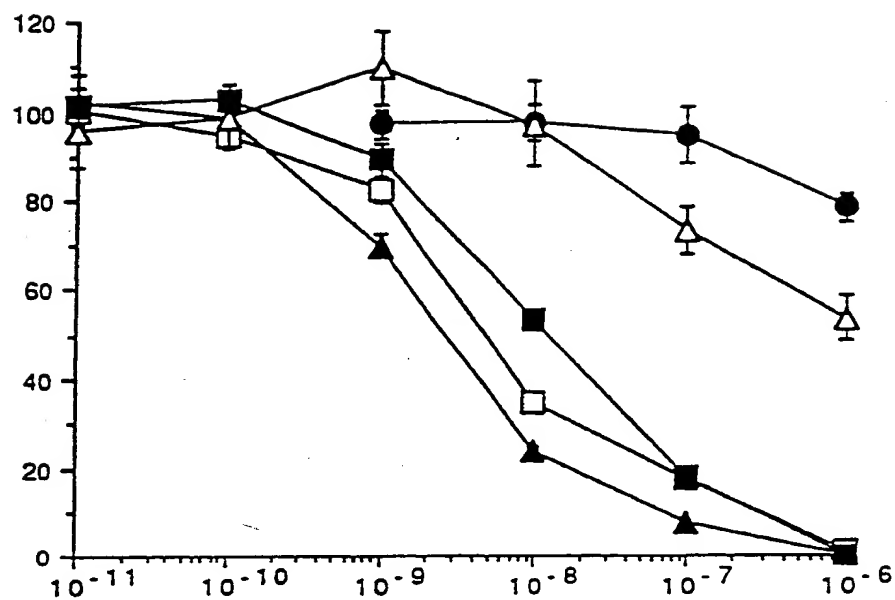


Fig. 1

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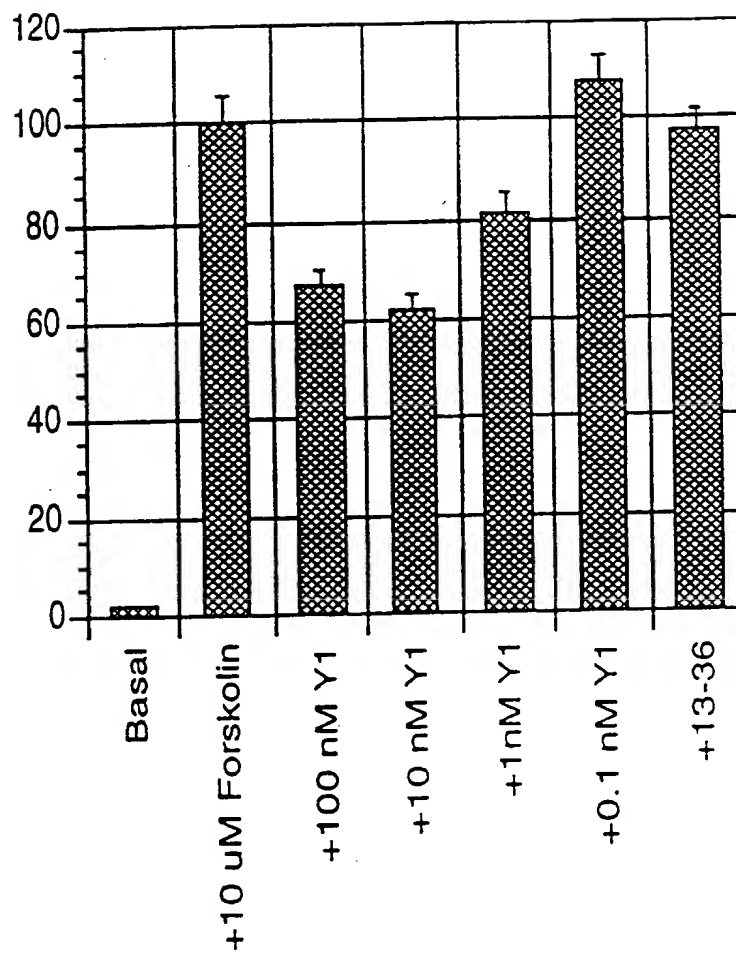


Fig.2

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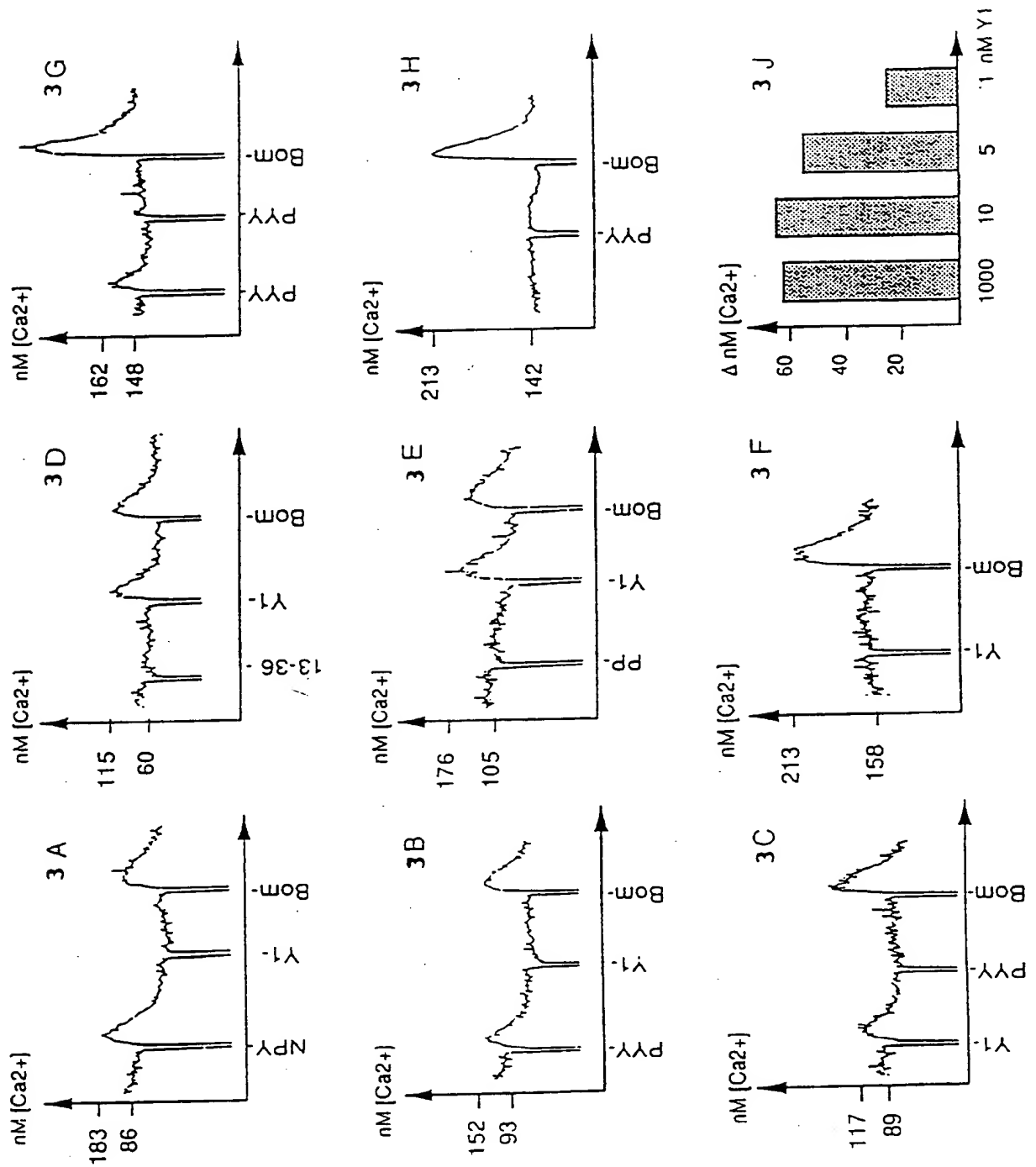


Figure 3.

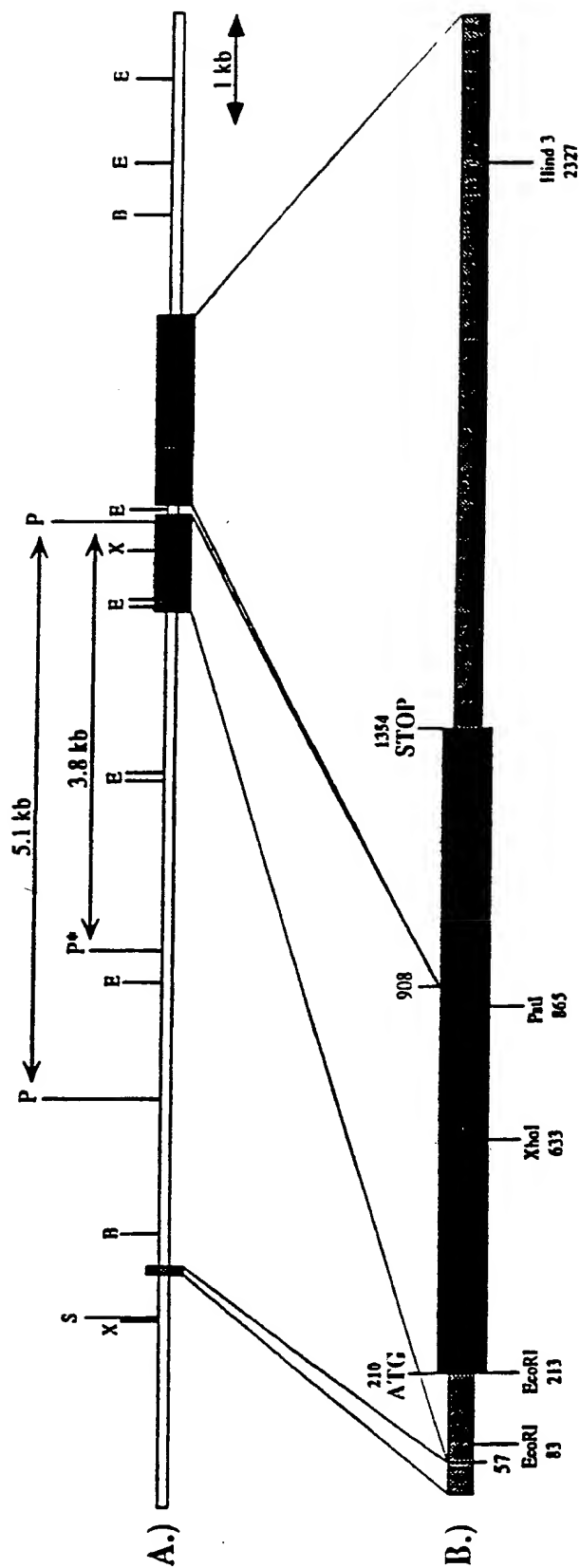


FIG. 4

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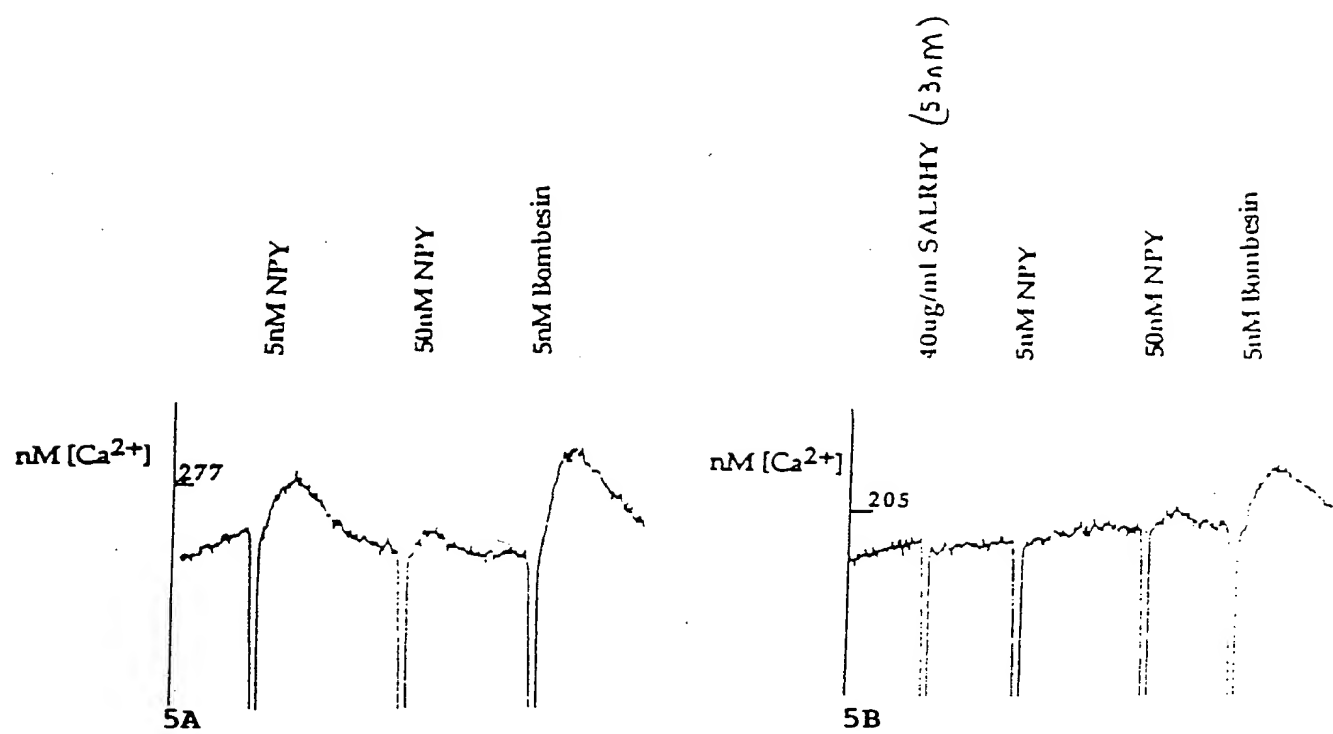


FIG. 5

A. CLASSIFICATION OF SUBJECT MATTERInt. Cl.⁵ C12N 15/12 C07K 13/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

WPAT, CHEMICAL ABSTRACTS DATABASES KEYWORDS : NEUROPEPTIDE, Y, Y₁, RECEPTORDocumentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU : C12N 15/12 C07K 13/00; BIOTECHNOLOGY ABSTRACTS

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)

WPAT, CHEMICAL ABSTRACTS, BIOTECHNOLOGY ABSTRACTS KEY WORDS AS ABOVE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	SHEIKH S P et al "Y ₁ and Y ₂ receptors for neuropeptide Y" FEBS LETTERS, published March 1989 volume 245 pp 209-214.	1-13
A	FR.A, 2628750 (INSTITUT PASTEUR) 22 September 1989 (22.09.89) See page 2 and claim 1.	1
A	EP.A, 355793 (MERRELL DOW PHARMACEUTICALS INC) 28 February 1990 (28.02.90) See pages 2 and 3 and examples.	1

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.*** Special categories of cited documents :**

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search
15 February 1993 (15.02.93)Date of mailing of the international search report
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DOCUMENTS CONSIDERED TO BE RELEVANT		
C(Continuation).		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
A	EP,A, 355794 (MERRELL DOW PHARMACEUTICALS INC) 28 February 1990 (28.02.90) See pages 2 and 3 and examples.	1
A	Feth et al "G-Protein coupling and signalling of Y ₁ -like neuropeptide Y receptors in SK-N-MC cells" Archives of Pharmacology (1991) Volume 344 pages 1-7.	1
A	Sheikh S.P. et al "Binding of Monoiodinated Neuropeptide Y to Hippocampal Membranes and Human Neuroblastoma Cell lines" Journal of Biological Chemistry issued 25 April 1989, vol 264 pages 6648-6654.	1
A	Aakerlund L et al "Y ₁ receptors for neuropeptide Y are coupled to mobilization of intracellular calcium and inhibition of adenylate cyclase" FEBS LETTERS published January 1990, volume 260 pages 73-78.	1